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原　　著

Scanning Electron Microscopic Study of Intravenous Fat Emulsions

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Since a clinically usable fat emulsion with soy lecithin was first developed in our clinic 30 years ago[2], six commercial 10% or 20% fat emulsions have been produced in Japan. It is emphasized that fat particles above 1 micron in size are not contained in these emulsions[3)4)5)]. However, many physicians are still anxious about the enlargement of fat particles and/or the stability of the emulsification during the manufacturing process and during storage in the hospital pharmacy. They fear fat embolism in the lung or brain from the intravenous administration of fat emulsion, although no clinical reports of fat embolism have appeared.

Among the studies on the stability of fat particles in these emulsions, there have been only a few with transmission electron microscopy, but exact observation of fat particles was not possible even with this method, because of the difficulty of keeping the particles unchanged in oil-in-water emulsion. Recently malachite green has been used by TEICHMAN et al[9)] as a stabilizer in the fixation of lipids, which were lost during conventional fixation for electron microscopy.

In the study reported here, scanning electron microscopy was chosen for the observation of flawless globular forms of fat particles ; the specific fixation technique with malachite green was established, and the homogeneity of the six fat emulsions is discussed in relation to the natural 3-dimensional figures of fat particles.

Materials and methods

Six commercial fat emulsions preserved at 4°C in cold storage were chosen for these observations. The emulsions of soybean oil with egg lecithin are 10% Intralipid made in Sweden and 10% Intrafat (Daigo Pharm, Co.), and 10% and 20% Intralipos (pilot samples,

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Table 1. Combination of fixatives and results of observations

	Methods of fixation			Results of staining	
	Prefixation (15 min)	Washing in buffer	Postfixation (15 min)	Agglutination	Retaining of larger particles
A.	GL	−	−	no fixation	
B.	OS	−	−	##	×
C.	MG	−	−	no fixation	
D.	GL	+	OS	##	×
E.	OS	+	GL	##	×
F.	GL−OS	−	−	##	×
G.	OS	+	MG	##	×
H.	GL	+	MG	no fixation	
I.	MG	+	OS	+	△
J.	MG	+	GL	no fixation	
K.	GL−MG	−	−	no fixation	
L.	OS−MG	−	−	minimum	○
M.	GL−MG	+	OS	minimum	○
N.	OS	+	GL+MG	##	×
O.	OS−MG	+	GL	minimum	○
P.	GL	+	OS+MG	minimum	○
Q.	MG	+	OS+GL	+	○
R.	OS−GL	+	MG	##～##	×
S.	OS−MG−GL*	−	−	minimum	○

GL: 2% glutaraldehyde, PH 7.2 phosphate buffer
OS: 1% osmium tetroxide, pH 7.2 phosphate buffer (sucrose added)
MG: 1% malachite green, pH 7.2 phosphate buffer (sucrose added)
−: mixing equivalent volumes
*: OS/MG−GL=1/1

The Green Cross Corp.) made in Japan. Venolipid (pilot samples Morishita Pharm. Co.) is an emulsion of soybean oil with soy lecithin, and Fatgen-D (Dainippon Pharm. Co.), which is produced according to our original idea, is made from sesame oil with soy lecithin in Japan.

Aliquots of 0.2 ml of these fat emulsions preserved in cold storage were put into sterile test tubes, diluted 25 times with 4.8 ml of cold saline, mixed very gently and kept in ice water before the following examinations :

Whatman 2 qualitative filterpaper was cut into narrow strips 5 by 20 mm. These pieces were prepared with defatted forceps and scissors to prevent contamination by the hands of the operator. The paper strip was immersed in the diluted fat emulsions and pulled out quickly. Fat particles adsorbed on the filterpaper were examined. Filterpaper immersed in saline alone was used as a control. The fixative solutions, osmium tetroxide, glutaraldehyde and malachite green, were freshly prepared in phosphate buffer containing sucrose at pH 7.2. These samples were fixed for electron microscopy by various methods, as shown in Table 1. Each fixation was performed for 15 min at 4°C.

The samples were subsequently dehydrated in a graded series of ethyl alcohols, and substitution with isoamyl acetate was omitted. After critical point drying with liquid carbon dioxide by Critical point dryer (Hitachi, Japan), the samples were glued to a small sample table with white cemedine, dried for one hour and stored in a vacuum of 0.05 torr. The samples were then coated about 300 Å thick with gold vapour without carbon by Ion coater (Eiko IB-3 model, Japan) and observed by Scanning electron microscope (Hitachi S-310 desk model, or Hitachi HFS-2S model, Japan).

Results

The meshes of the network of fibers in the filterpaper could easily catch the larger particles, which had escaped from the smooth surface. However, beam damage easily occurred on the filterpaper, especially with the Hitachi HFS-2S model electron microscope in which a single particle can be observed at 40,000 magnification. On the contrary, beam damage of the samples was scarcely observed at 4Kv of accelerating voltage in a Hitachi S-310 desk model electron microscope. So, the latter was used for the following observations in this study.

In the control group, no particles on the fibers of the filterpaper were observed with any fixatives or methods, as shown in Fig. 1. This fact indicates that none of the fixative

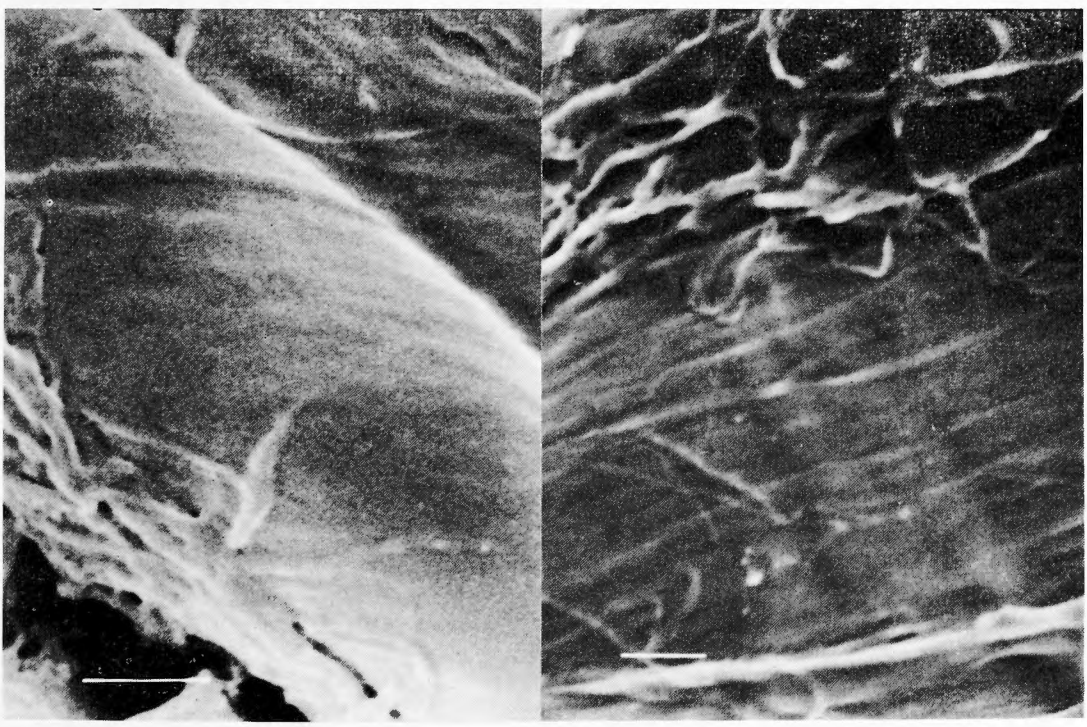

Fig. 1. Control background ; a) the filterpaper immersed in saline alone after fixation of combination method with glutaraldehyde-malachite green and osmium tetroxide. b) Fixation with glutaraldehyde or malachite green alone showed the same figure. White line in each figure indicate 1μ.

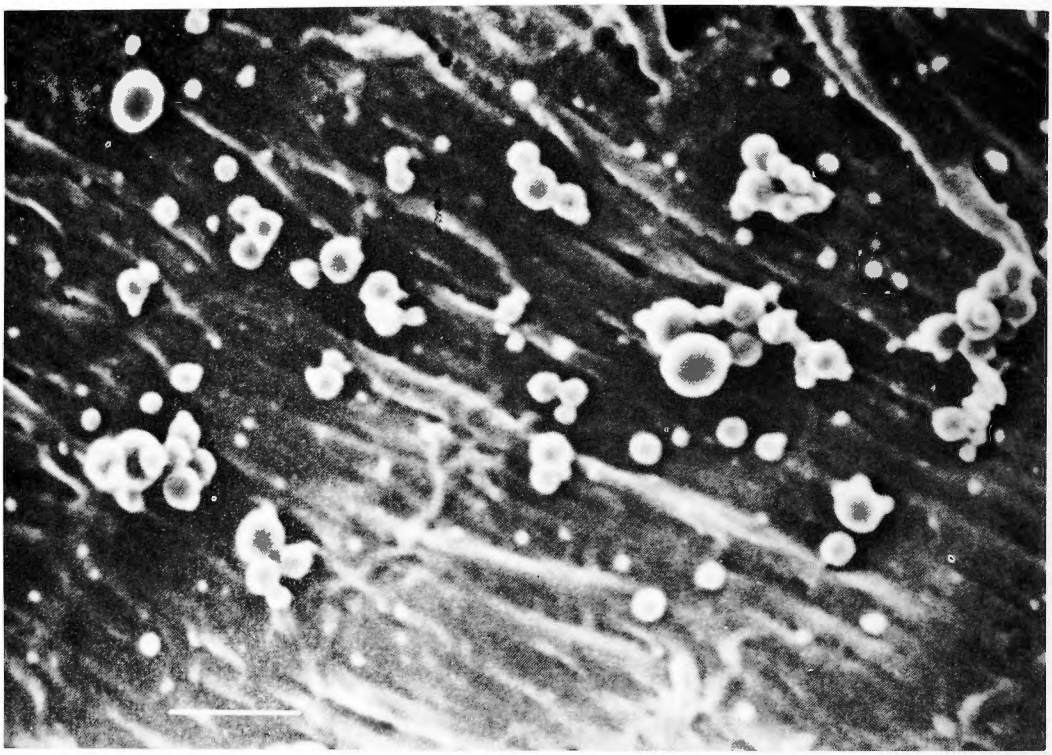

Fig. 2. Agglutination of fat particles was observed at fixation with osmium tetroxide alone or after glutaraldehyde fixative.

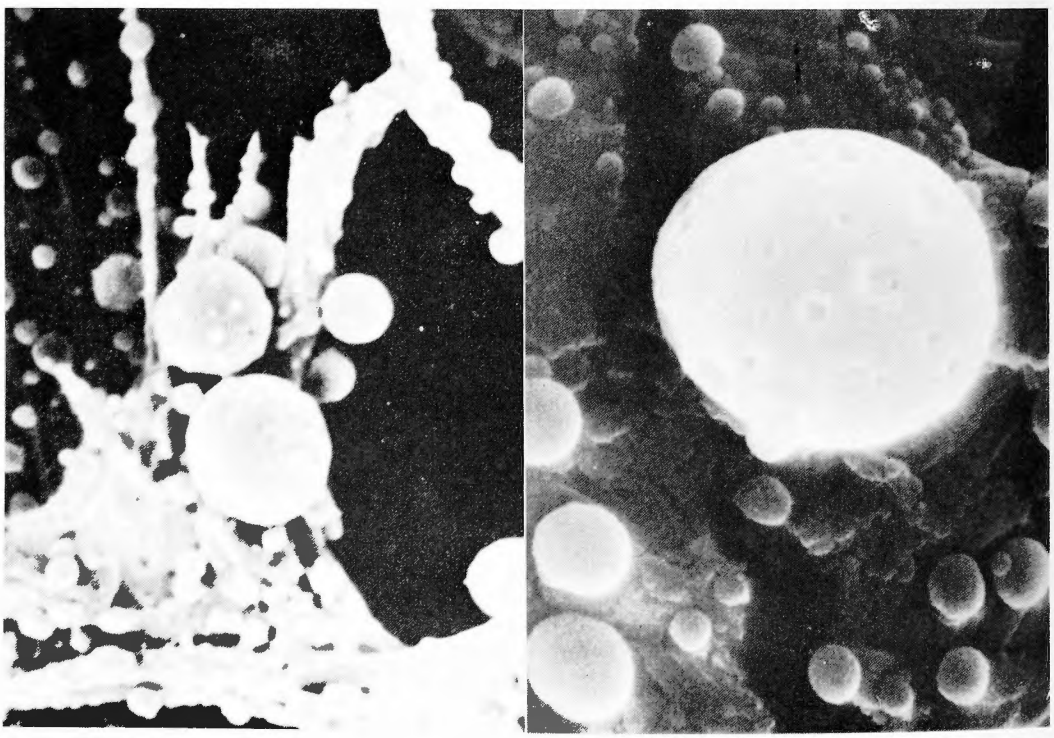

Fig. 3. a) Osmication after pretreatment with malachite green showed some dents of the surface of particles like a golf-ball. b) Same figure in 40,000 magnification.

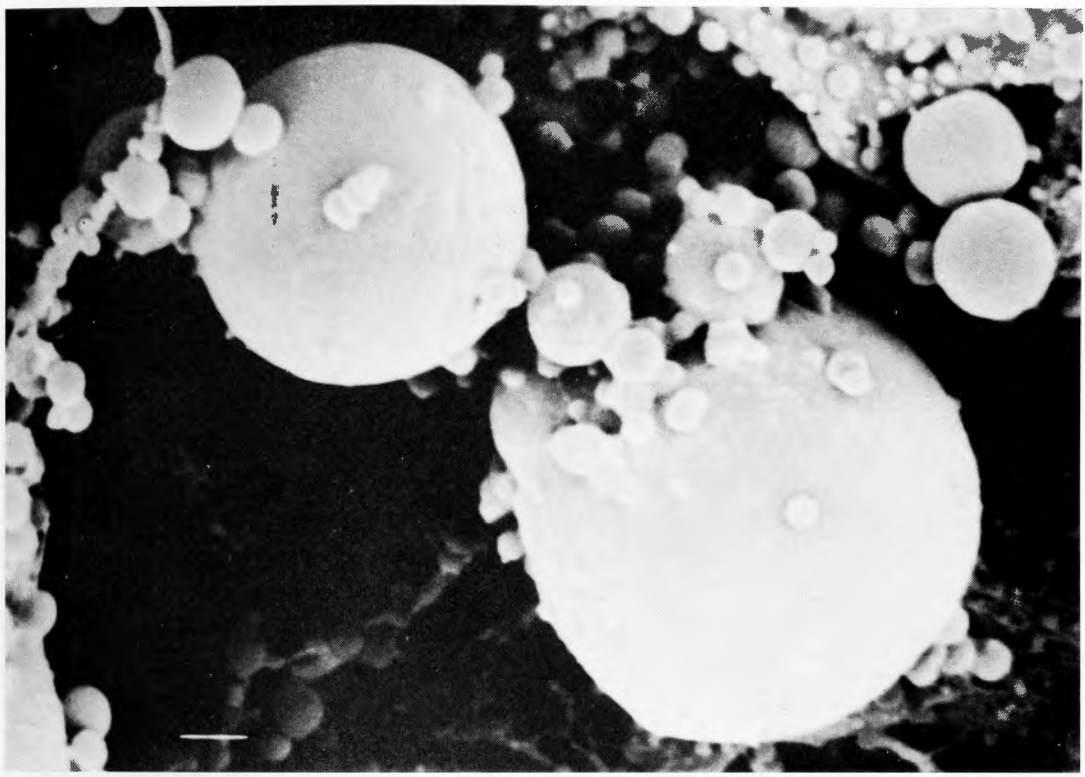

Fig. 4. Large fat particles such as 4 to 5 micra in diameter were observed in 20% Intralipos.

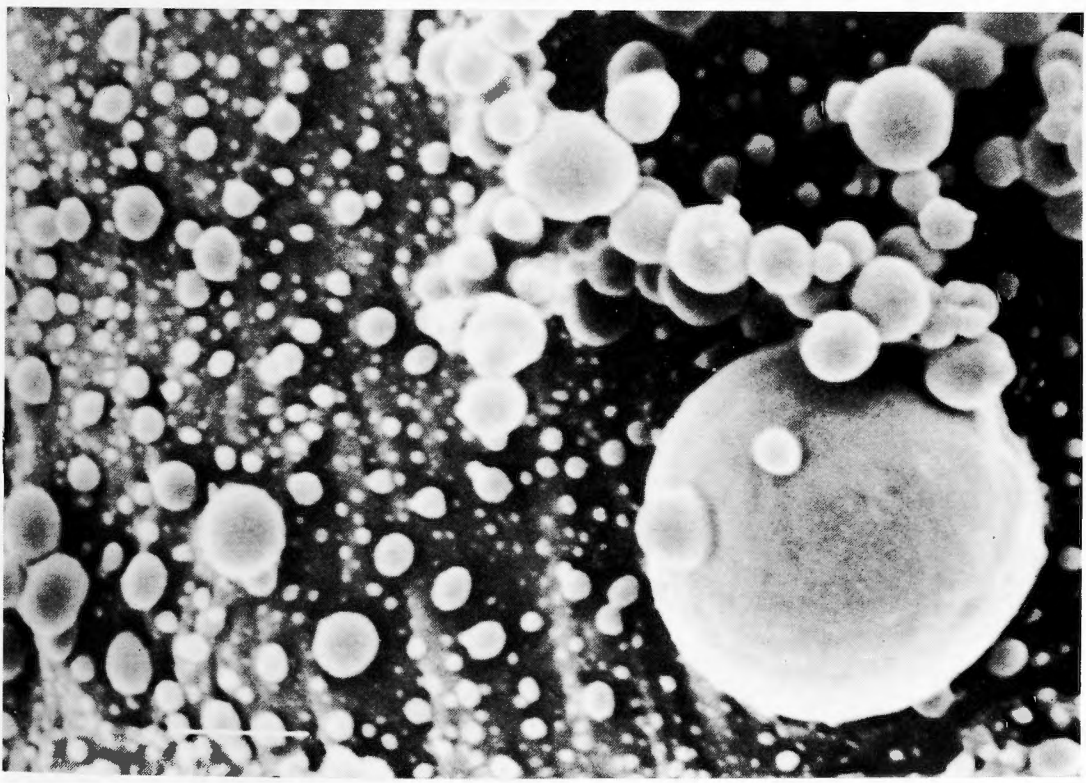

Fig. 5. Some larger fat particles observed in 10% Intralipos.

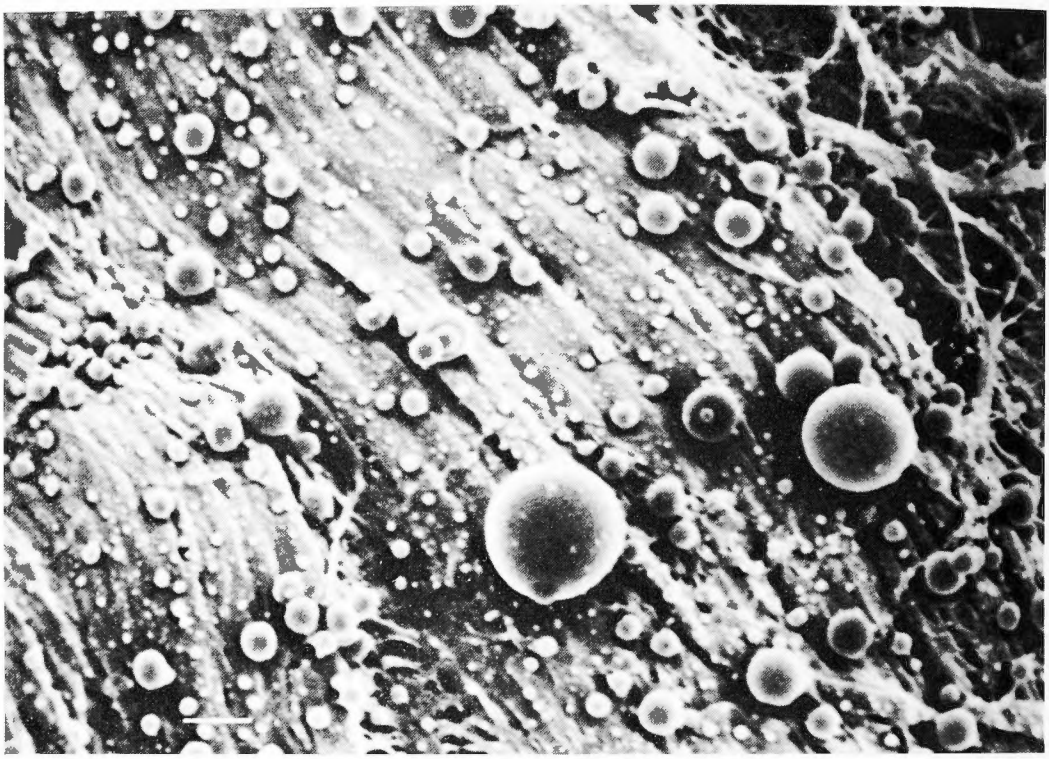

Fig. 6. Some larger fat particles observed in 10% Intralipid.

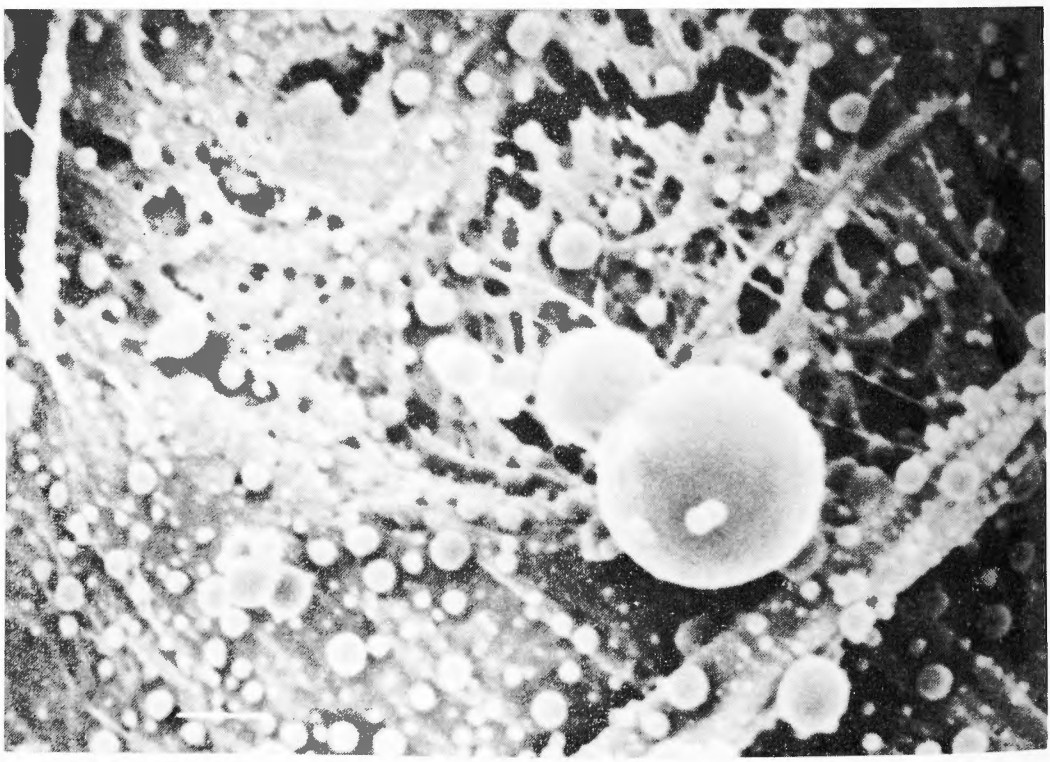

Fig. 7. Some larger fat particles observed in 10% Intrafat.

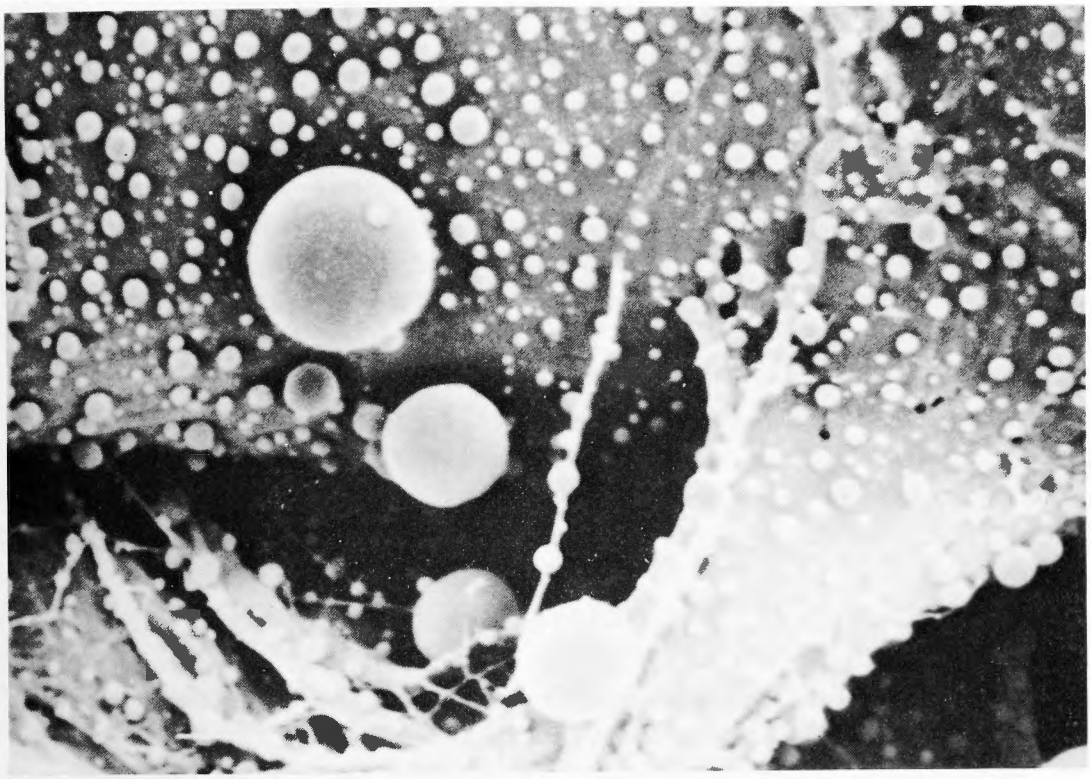

Fig. 8. Some larger fat particles observed in 10% Venolipid.

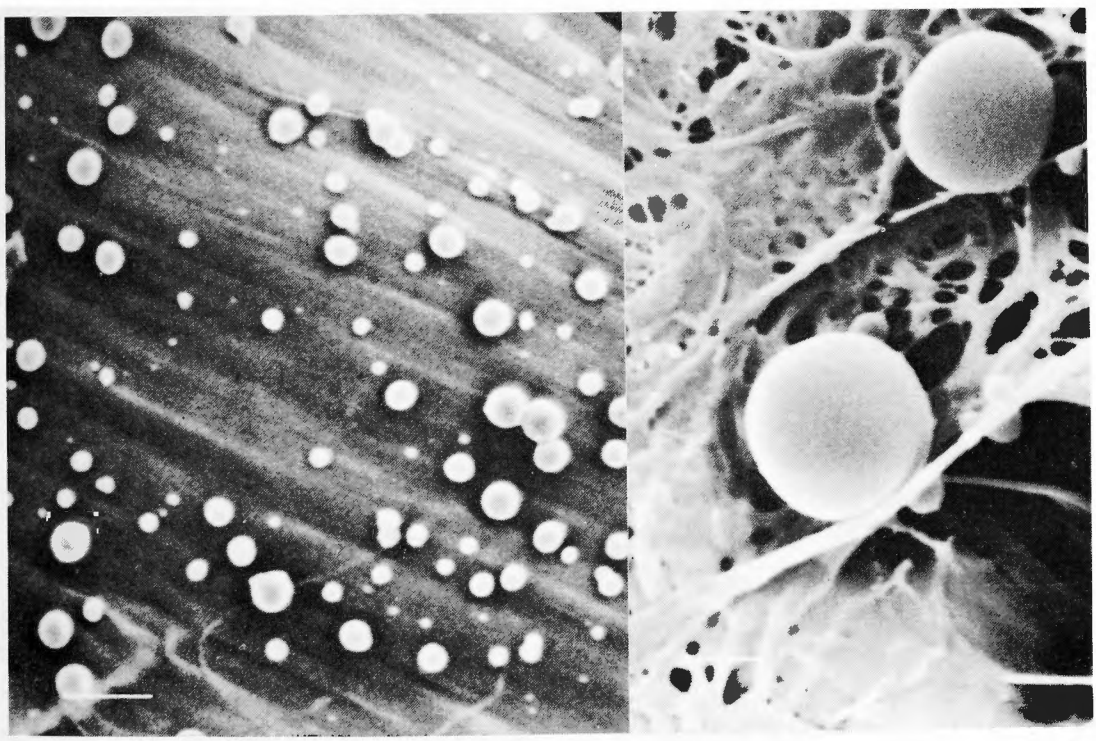

Fig. 9. a) Fat particles observed in Fatgen-D.
b) Larger particles were very rarely observed in Fatgen-D.

produced any particles as artifacts.

When fixation was with 2% glutaraldehyde or 1% malachite green alone, neither of which has a fixing effect, no particles were observed.

With 1% osmium tetroxide alone, agglutination among fat particles was often observed and some particles over 1 micron in diameter were unsatisfactorily fixed, as shown in Fig. 2. Prefixation with 1% osmium tetroxide hindered rather than helped the fixation of fat particles and the image was sparse, especially with large particles. Malachite green after osmication could fix some particles, but sparsely and with agglutination. Osmication after pretreatment with malachite green only showed some dents in the surface of the particles, like a golfball, as shown in Fig. 3, although fixing condition was thought to be well.

A combination method of 2% glutaraldehyde and 1% osmium tetroxide induced relatively good fixation, but agglutination of some particles was observed. Particles about 1 micron in diameter were fixed poorly and sparsely, but those over 1 micron were sometimes fixed. So, this fixing combination, used routinely for biological materials, was inadequate for the observation of fat particles.

The formulation with 2% glutaraldehyde containing 1% malachite green reduced agglutination of fat particles to a minimum and enabled direct measurement of each particle. The combination of glutaraldehyde-malachite green and osmium tetroxide resulted in the best fixation of fat particles as in methods M and S.

On the basis of these results, methods L, M, O, and S were thought to be preferable as fixatives for electron microscopic observation of fat particles in intravenous fat emulsions.

However, 1% malachite green in phosphate buffer produces precipitates even a in cool room within 24 hours, so it must be prepared just before use and be applied as soon as possible.

In the two-step methods, the sample must be washed in phosphate buffer and sucrose for 15 min before postfixation, and imperfectly fixed particles may be carried away. So, a one-step fixation with a mixture of glutaraldehyde-malachite green and osmium tetroxide was used for comparing fat emulsions, and the following results were obtained :

1) In 20% Intralipos, large fat particles, up to 4 to 5 micra in diameter were not rare, as shown in Fig. 4.

2) 10% fat emulsions such as Intralipos, Intralipid, and Intrafat showed the same findings, as shown in Figs. 5, 6, and 7. Large particles more than 1 to 3 micra and much smaller particles were also observed. The spectrum of the size of fat particles was broad.

3) Venolipid was between groups 1) and 2), as shown in Fig. 8.

4) Fatgen-D had homogeneous fat particles, almost none more than 1.5 micron in diameter and no very small particles under 0.1 micron (Fig. 9).

Discussion

The size of the fat particles in clinically used fat emulsions was previously examined by transmission electron microscope[1)6)], but deformity of the fat particles could not be

avoided. The scanning electron microscope permits observation of spherical particles, but the previous fixing methods are inadequate for fatty materials. Fat particles fixed by osmication appeared only as round or oval black shapes on transmission electron microscopy. Intralipid and Intrafat were observed by drying at room temperature on copper grids for transmission electron microscopy[1)6)], but the fat particles dried by this method looked like overturned dishes by of scanning electron microscopy. As Venolipid was embedded in Epon resin and fixed by osmication only, the figure in transmission electron microscopy was one of beehive-like shells[7)], because of the dense particles of undiluted fat emulsion and loss of fatty elements due to repeated washing with a graded series of ethyl alcohols. Osmium tetroxide alone has a fairly good fixing action for fat particles, but some agglutination and incomplete fixation of the particles were observed.

Intravenous fat emulsions are made up of triglycerides of oleic and linoleic acids emulsified by lecithin. TEICHMAN et al used 20 different cationic dyes incorporated into a glutaraldehyde fixative to preserve fatty material within mammalian spermatozoa and found that three dyes belonging to the phenyl methane (malachite green) and xanthene (pyronine B and Y) groups gave the best results with staining specificity for the fatty acid components[9)]. Each of these dyes contains two tertiary amine group, the substituted radical being either a methyl or an ethyl group and preferentially binds to lipid elements. They also demonstrated that malachite green specifically stains phospholipids, cholesterol, fatty acids (oleic, myristic, stearic, palmitoleic), glyceryl tripalmitate and beta-lipoprotein, but not glycerol, glucose, sucrose, albumin, etc[10)]. POURCHO et al demonstrated that incorporation of malachite green into a glutaraldehyde fixative results in enhanced staining of a number of cellular elements, such as ribosomes, myofilaments and lipid inclusions[8)].

The mechanism of this staining is not known. In addition to its function as a dye, malachite green stabilizes lipids soluble in aqueous glutaraldehyde. Preservation of emulsified fat particles may require glutaraldehyde as a cross linking agent between the tertiary amine groups of the dye and phospholipids. Following treatment with osmium tetroxide it presumably forms a double coordinate link across one of the reactive quinone ring double bonds within each dye molecule, and renders the malachite green-lipid complex stable, insoluble and electron dense. The combination of glutaraldehyde and malachite green plus osmium tetroxide permits visualization of flawless fat particles in the scanning electron microscope.

To support fat particles, filterpaper was chosen because of its ease of manipulation and the uniform strips obtained from one paper. For sampling from fat emulsions, decomposition by chemical and physical manipulation, such as heating, stirring, centrifugation or compression, must be avoid as much as possible. So, the filterpaper must be immersed very gently in the diluted fat emulsions and a thin layer of fat particles fixed to the paper. Dye and fixatives react directly and rapidly on the paper without physical change.

Although the method of prefixing with glutaraldehyde and postfixing with osmium tetroxide is generally used in biological materials for scanning and transmission electron

microscopy, the reverse method is selected for fixation of fatty materials. In both methods, however, washing in phosphate buffer at pH 7.2 is interposed for 15 min between the two fixing procedures, and two-step methods seem to wash out large fat particles. So, the combination method where osmium tetroxide is finally added to the glutaraldehyde-malachite green solution may be preferred as simultaneous fixation, because osmium tetroxide is expensive.

The fat emulsions examined here are some what different in regard to the use of lecithin as a stabilizer, to the steps of the emulsifying process and to the method of sterilization. Intralipid, Intrafat and Intralipos 10% fat emulsions gave similar results, because of the same system of production. A new fat emulsion, Venolipid, showed more irregularity of fat particles, and 20% Intralipos contained several large particles up to 4 to 5 micra in diameter. Our previously developed fat emulsion, Fatgen-D, had relatively uniform particles. This difference might be due to the careful two-step homogenization by a circularting pressure system avoiding ultrasound technqiues.

Observation of fat particles in fat emulsions by scanning electron microscopy is very easy and rapid, and may be useful in the quality control of fat emulsions in the pharmaceutical in the industry.

Summary

Fat particles in clinically used intravenous fat emulsions could be observed as flawless globular forms by scanning electron microscopy.

In complete staining and agglutination of particles occurred with fixation by osmication alone. The incorporation of malachite green into a glutaraldehyde fixative resulted in stability of fat particles, and then treatment with osmium tetroxide rendered fat particles electron dense. One-step fixation with a combination of glutaraldehyde-malachite green and osmium tetroxide was the most effective avoided washing out of large particles.

Six commercial intravenous fat emulsions were compared by this staining method. Some fat emulsions were found to contain particles larger than 1 micron in diameter, while Fatgen-D had more uniform particles than the other fat emulsions.

This method may be useful in the quality control of fat emulsions during pharmaceutical manufacture.

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和文抄録

静脈注射用脂肪乳剤の走査電子顕微鏡的観察

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脂肪乳剤の静脈内投与で，専ら懸念されている副作用としては，脂肪粒子の安定性とそれに関連する脂肪栓塞に関する問題であろう．現在市販の脂肪乳剤は1μを超えないとされているが，いずれもその乳化された脂肪粒子の大きさに関する研究は，Svedberg の遠沈法や透過電子顕微鏡による不確実な方法でしか行われていない．われわれは乳化された脂肪粒子の大きさを立体的に観察するために，走査電子顕微鏡を用いて検討した．

〔**支持体の検討**〕 乳化態となっている脂肪粒子の変性や変化を防ぐため，加熱，攪拌，遠沈，加圧等の物理的，化学的操作をできるだけ介在せしめないようにした．そのため，適当に希釈した脂肪乳剤に，沪紙を浸して薄い乳剤層を形成させ，これに直接固定剤を作用させる方法が最もよいことが判明した．

〔**固定法の開発**〕 静脈注射用脂肪乳剤は，中性脂肪としてオレイン酸とリノール酸が主体であり，界面活性剤としてレシチンが用いられている．この脂肪酸とリン脂質に共通の固定作用のある malachite green を併用する方法を考案し，従来の O_s 酸と glutaraldehyde との20種類の組合せを検討した結果，O_s 酸と glutaraldehyde に malachite green を溶解した液による同時固定が最も優れていることが判明した．

〔**各種脂肪乳剤の観察**〕 ① 20%大豆油・卵黄レシチン乳剤は，脂肪粒子の均一性は極めて不良で，数μに及ぶものがかなりの頻度に観察された． ② 10%大豆油・卵黄レシチン乳剤は2～3μに及ぶものも少なくなかったが，極めて小さな脂肪粒子もあり，その粒子直径の範囲は大きかった． ③ 10%大豆油・大豆レシチン乳剤はこの中間に位置した．

④ しかし，われわれが開発に関与したゴマ油・大豆レシチン乳剤は粒状性が均一化しており，1.5μを超えるものは極く稀であり，また極めて小さい 0.1μ 以下のものは殆んどなかった．

〔**結論**〕われわれは静脈注射用脂肪乳剤の走査電子顕微鏡的観察のための固定法として， malachite green を併用した Os 酸，glutaraldehyde 同時固定法が最も良いことを見い出した．

この方法を用いて，静脈注射用乳脂乳剤6剤の脂肪粒子の状態を観察し，従来の方法では検出されなかった大きい粒子を捕えることが出来た．この方法は，試料の固定から観察まで半日で十分可能であり，経済的にも安価であり，脂肪乳剤の品質管理にも利用できるとともに，生体における脂肪球の追跡にも応用できるものと思われる．